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Paper Title:

Feasibility study on the use of in vivo microCT to follow up ectopic 3D mineralized tissue formation

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# Feasibility study on the use of *in vivo* microCT to follow up ectopic 3D mineralized tissue formation

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## Aims

Bone tissue engineering (TE) is a multidisciplinary field that aims to repair large bone defects by the production of biological spare parts. The focus lies on the design and manufacturing of TE constructs that combines several "raw materials" such as carrier structures (scaffolds), osteogenic (bone forming) cells and growth factors. Despite the fact that successful results have been published, existing bone TE strategies still suffer from unpredictable and qualitatively inferior results, thus hampering clinical translation [1, 2]. This low repeatability is caused by several factors, including insufficient insight in the 3D mechanisms of *in vivo* tissue formation depending on the TE construct raw materials. Innovations in imaging techniques are fundamental to fully understand the complex 3D events during tissue formation.

In this study, the aim was to investigate the feasibility of using *in vivo* microfocus X-ray computed tomography (*in vivo* micro-CT) to follow up mineralized tissue formation in TE constructs. We present here its potential as well as the constraints for the visualization and quantification of the formation of mineralized tissue in different ectopically implanted TE constructs in nude mice.

## Method

### TE constructs

To assess the effect of TE construct raw materials on the quality of the *in vivo* microCT datasets, several combinations of these raw materials were evaluated in an ectopic nude mouse model at different time points post implantation, ranging between 1 day and 8 weeks. Three clinical grade calcium phosphate (CaP)-collagen composite scaffold types with distinct differences in CaP and collagen content and structure were used, namely BioOss®, NuOss™ and CopiOs®. Scaffolds of different sizes, made from these materials, were coated with a growth factor or left uncoated, and subsequently seeded with human periosteal derived cells (hPDCs). The constructs were kept in growth medium overnight, allowing cell attachment, and implanted subcutaneously on the back of a nude mouse the next morning.

### *In vivo* and *ex vivo* micro- and nanoCT

To determine the scaffold material characteristics and to quantify the morphology, the implants were scanned with a Phoenix NanoTom S system [GE, Germany] prior to implantation. The isotropic voxelsize was 2.5 µm and the system was equipped with a Diamond/Tungsten target, operated at a voltage of 60 kV and a current of 210 µA, a 1mm Al filter was applied and 2400 images were taken per scan. The exposure time was 500 ms, resulting in a scanning time (in fast mode) of 20 minutes. Reconstruction was performed with dedicated software [GE, Germany].

*In vivo* microCT scanning was done at different time points post implantation, ranging between 1 day and 8 weeks, with a Skyscan 1076 system [Bruker microCT, Kontich, Belgium] at 9 µm voxelsize. A 1 mm Al filter was applied with a source voltage of 50 kV and a current of 100 µA. The rotation step was 1° and we used a frame averaging of 1. The scan time was about 12 minutes. To reduce movement of the implant during scanning due to the animal's breathing and heartbeat, we have mounted the mouse on its back in the animal bed of the CT device. Reconstruction was performed using NRecon [Bruker microCT, Kontich, Belgium].

At week 4 or 8, the animals were sacrificed and the TE constructs were harvested, immediately fixed in 4% paraformaldehyde and finally scanned again with the Phoenix NanoTom S system, using the same scanning parameters as for the implants, for the comparison between the *in vivo* and *ex vivo* acquired CT datasets.

**Image processing and analysis, and 3D visualization**

For image processing and analysis, we used CTAn [Bruker microCT, Kontich, Belgium]. Regions of interest (ROIs) were manually drawn on the *in vivo* CT and automatically (using an in-house developed MatLab routine) drawn on the *ex vivo* CT datasets. For the implants, the CaP and collagen content, and their thicknesses were determined after multilevel Otsu segmentation (3). For the explants and the *in vivo* microCT datasets, the amount of mineralized material was quantified after manual segmentation. CTVox [Bruker microCT, Kontich, Belgium] was applied for 3D visualization.

## Results

**Influence of the scaffold material and movement on the image quality**

The three scaffold types showed distinct differences in CaP and collagen content, structure and distribution (Fig. 1 and 2). CopiOs® contained a low amount (~ 2 volume%) of CaP granules, while BioOss® and NuOss™ contained significantly more CaP granules (about 25 volume% and 15 volume% respectively).

After implantation, because of the limited mechanical strength, the cylindrical scaffolds are compressed, causing a significant decrease in space between the CaP granules. As a result of this small space (between 20 µm and 140 µm after implantation) and the limited spatial resolution, both for NuOss™ and BioOss® it was not possible to detect newly formed mineralized tissue using *in vivo* microCT (Fig. 3). For CopiOs®, the larger space between the CaP granules (ranging between 20 and 600 µm) as well as the smaller CaP content allowed to visualize the "mineral content", i.e. both newly formed mineralized tissue and CaP granules. No discrimination could be made between the CaP granules and the newly formed mineralized tissue (Fig. 3). Hence the scaffold composition and structure strongly influences the detectability and image quality of *in vivo* microCT.

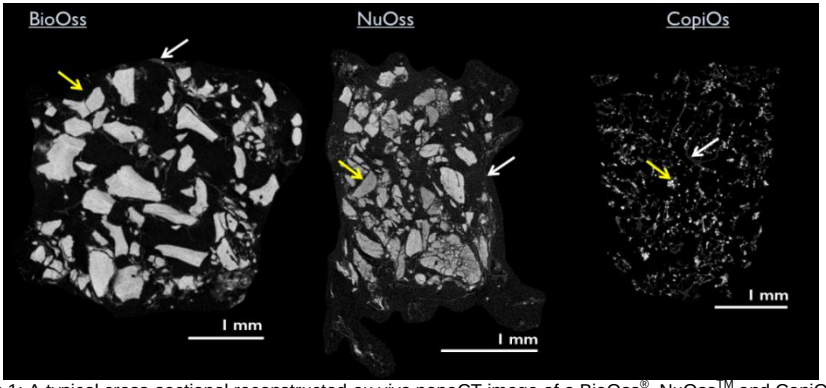


Fig.1: A typical cross-sectional reconstructed *ex vivo* nanoCT image of a BioOss®, NuOss™ and CopiOs® scaffold. The light grey phase (yellow arrow) is the CaP, the darker grey (white arrow) is the collagen network.

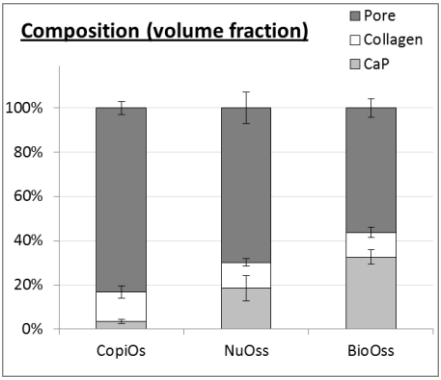


Fig.2: *Ex vivo* nanoCT based composition (volume fraction of CaP, collagen and pore space) of the different types of scaffold material. Both the CaP and the pore volume fraction were significantly different for the three scaffold materials types.

Apart from the scaffold material-related errors in image quality, also sample movement introduced image artefacts (Fig. 3 – NuOss™). We tried to limit this by fixing the animal on its back during scanning. Again due to the limited space between the CaP granules, for BioOss® and NuOss™ these movement artefacts were detrimental for the image quality (Fig. 3), while for CopiOs® small movements still allowed visualization of the mineral content.

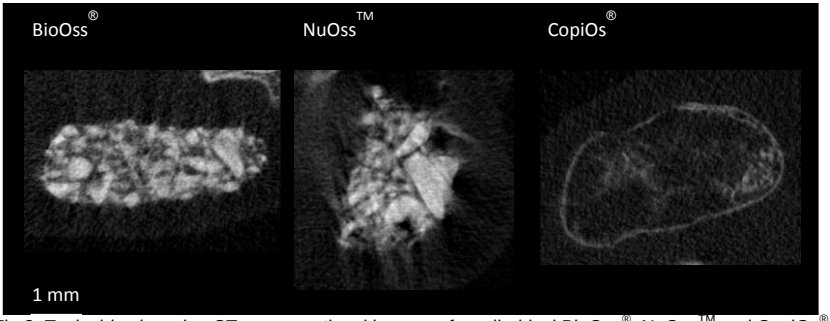


Fig.3: Typical *in vivo* microCT cross-sectional images of a cylindrical BioOss®, NuOss™ and CopiOs® explant after 3 weeks of implantation.

**3D quantification of the dynamics of mineralized tissue formation**

Figures 4 and 5B show typical images and the 3D quantification respectively of the mineral content present in CopiOs® constructs (using different seeding protocols) during 8 weeks of ectopic implantation. A decrease in construct size as well as mineral volume was observed (Figs. 4 and 5) after 8 weeks of implantation. Both for construct 1 and 2, however, a clear increase in density of the mineral content was seen (Fig. 5). For construct 2, even bone spicules were formed after 8 weeks of implantation (confirmed by histology).

Comparison between the *in vivo* and *ex vivo* CT-based visualization (Fig. 6A) and quantification (Fig. 6B – n=5) of the same TE construct showed a significant difference in mineral content. *In vivo* microCT overestimated the mineral content, and this error was strongly ($R^2$ = 0.98) dependent on the volume of the mineral content (labels in Fig. 6B represent the overestimation compared to the *ex vivo* CT results). The lower the amount of mineral content present, the larger the relative overestimation was. This was amongst others caused by the strong presence of noise in the *in vivo* microCT images (Fig. 3).

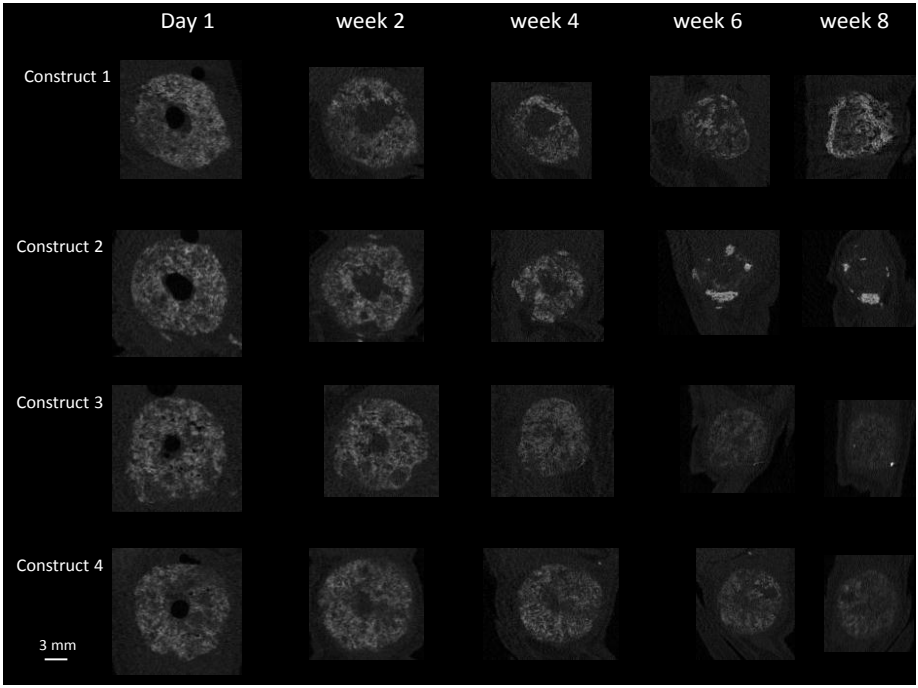


Fig.4: Overview of typical cross-sectional *in vivo* microCT images of 4 different donut-shaped TE constructs (different seeding protocols were used), taken at day 1 and after 2, 4, 6 and 8 weeks.

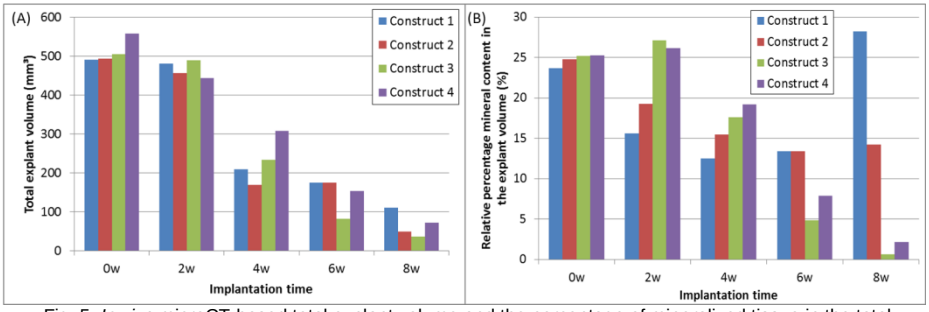


Fig. 5: *In vivo* microCT-based total explant volume and the percentage of mineralized tissue in the total explant volume for the 4 different TE constructs (different seeding protocols were used), determined at day 1 and after 2, 4, 6 and 8 weeks.

Although being on a limited amount of samples, correlating the *in vivo* and *ex vivo* CT-based results allowed to define a linear equation (Fig. 6B) predicting the *ex vivo* CT-based mineralized tissue volume based on the *in vivo* CT results. Thus, for future studies, *in vivo* microCT can be used to dynamically follow up the mineral content in ectopically implanted CopiOs® constructs.

In the *ex vivo* CT images, at early time points of implantation a clear distinction could be made between the CaP granules and the newly formed mineralized tissue (Fig. 6A), mainly because

the CaP granules did not dissolve or degrade too much. In the *in vivo* microCT images, this was however not possible (Figs. 4 and 6A), hence making 3D quantification of newly formed mineralized tissue less accurate. A solution could be to subtract the amount of mineral content at day 0 (being CaP granules) from the mineralized tissue volume at consecutive time point analyses. As the *ex vivo* CT results showed CaP granule degradation over time, subtraction of the volume of CaP granules at day 0 from the mineral content at consecutive time point analyses would not be a solution as atime-dependent underestimation of the mineralized tissue volume would be made.

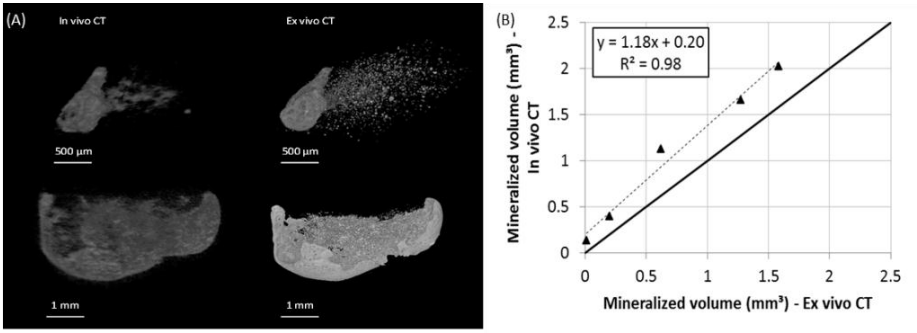


Fig.6: (A) Typical 3D visualizations of the *in vivo* CT and *ex vivo* CT dataset of the same TE construct (CopiOs® coated with a growth factor and seeded with hPDCs) and (B) correlation between the mineralized volume measured by *ex vivo* CT and by *in vivo* CT of the same TE constructs (n=5).

## Conclusion

Dynamic 3D imaging techniques are becoming more and more important to fully understand the complex 3D events during tissue formation. In this study, we investigated the feasibility of using *in vivo* micro-CT to follow up mineralized tissue formation in TE constructs. Several parameters influence the accuracy of the *in vivo* microCT results. Here, it was shown that the CaP content and the space between CaP granules play an important role. Given the implantation site, movement of the sample could not be eliminated during the imaging; however by fixing the animal on the animal bed during scanning it could be strongly limited.
For TE constructs containing a low amount of small CaP granules, quantitative dynamical follow-up of the mineral content in the explant during 8 weeks of implantation could be achieved when compensating for mineral volume-dependent errors. Discrimination between the CaP granules and the newly formed mineralized tissue was however not possible.

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